THE ROLE OF SPECIFIC SURFACE ANTIGENS IN CELL ADHESION. PART II. STUDIES ON EMBRYONIC AMPHIBIAN CELLS [1]

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Holtfreter (1943) first demonstrated that embryonic amphibian cells could be disaggregated by exposure to his standard solution (Holtfreter, 1931) at high pH. Townes (unpubl.) has utilized this technique to investigate the problem of cell affinities. The following account is based on these previous investigations.

If an amphibian gastrula or neurula is exposed to Holtfreter's solution at a pH greater than 9.6, the pigmented surface coat becomes soft and is partially dissolved. "Within a few minutes cracks appear in it; new disruptions follow, until the whole surface is broken up into small dark islets, surrounded by much larger white interfaces. A closer examination reveals that each islet is the centre of a group of cells, attached to it in a radial orientation" (Holtfreter, 1943; p. 292).

Under the action of the alkali, the surface coat, which is disrupted at the cell boundaries, retracts in the form of a black cap to the pole of each cell. This cap progressively decreases in size as pigment streams to the interior of the cell.

Within 20 minutes, the entire gastrula or neurula is a heap of dissociated spherical cells which can be separated from one another with a glass needle. If the solution is now removed and replaced with 2–3 changes of Holtfreter's solution at pH 7.8, the entire process is reversed.

The cells begin to adhere to one another immediately after the removal of alkali, and within 0.5–1 hour have formed a mass of irregular form. At 3–8 hours, partly depending on species used, the aggregate has rounded up into a smooth solid ball of cells in intimate contact. Ectoderm cells, at first randomly distributed within the aggregate, have migrated to the exterior and formed sheet-like patches among the endoderm, at the end of 24–72 hours. If sufficient ectoderm cells are supplied they will almost completely cover the aggregate. Mesoderm cells are chiefly found between the ectoderm and endoderm, with a few scattered among the endoderm cells (Townes, unpubl.). The surface coat is reconstituted and the entire mass is usually covered with pigment. During this period intercellular spaces appear due to the secretion of an interstitial fluid. It appears to be identical with blastocoel fluid, having the same property of reducing the mutual adhesiveness of the cells (Holtfreter, 1944). Smaller aggregates lack the interior intercellular spaces altogether.

If an aggregate is allowed to develop further, differentiation proceeds and may include development of neural folds, eyes, ears, beating heart, etc. (Townes, unpubl.).

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Holtfreter (1939a, 1939b) has shown that small pieces of ventral ectoderm and endoderm, brought into contact, at first cling together but later tend to separate into discrete masses. The self-isolation begins after 2 days in *Rana pipiens* and 3–4 days in *Ambystoma punctatum,* and is complete after 4 and 6–9 days, respectively. However, if mesoderm is combined with ectoderm and endoderm, self-isolation does not occur; the mesoderm behaves as a binding agent.

These tissue affinities are apparently not species-specific at early stages of development, and may be seen in practically any xenoplastic combination. For example Townes (unpubl.) has found that the ectoderm of *Rana pipiens* gastrula with the endoderm of *Ambystoma punctatum* or *Triturus torosus* will exhibit self-isolation. If, however, mesoderm is included, an aggregate is formed which holds together.

With these observations and experiments as a basis it seemed desirable to test the effects of antisera on the reaggregation, extending thus the previously reported (Spiegel, 1954) experiments on dissociated sponge cells.

EFFECTS OF ANTISERA ON REAGGREGATION AND SEGREGATION

Materials and Methods

Experimental induction of breeding activity. Fertilized eggs of *Rana pipiens* were obtained through ovulation by hypophysis injection, and artificial insemination, as described by Rugh (1948). *Triton alpestris* eggs were very kindly furnished by Dr. J. Holtfreter.

Preparation of protein extract. Ten grams of the plant enzyme papain were extracted with 500 ml. of 10% Holtfreter's solution (3.5 grams NaCl, 0.05 gram KCl, 0.1 gram $CaCl_2$, 0.2 gram $NaHCO_3$, per liter distilled water) for six hours at 4° C. (Spiegel, 1951). The solution was filtered, 300 mg. cysteine.HCl added, and the pH adjusted to 6.6 with 0.1 *N* NaOH and 0.1 *N* HCl. Stage 12 (Shumway, 1940) gastrulas, 3410 in number, were placed in an eight inch finger bowl, the bottom of which was lined with ¼ inch of 2% agar, and covered with 500 ml. of the papain solution.

After three hours, the two outer layers of jelly had dissolved; the eggs were washed twice with 10% Holtfreter's solution, and 500 ml. of 2.5% sodium thioglycolate (in 10% Holtfreter's solution, pH 8.1) added. In ten minutes the vitelline membrane and third jelly layer were dissolved. The eggs were washed with three changes of 0.65% NaCl (0.01 *M* phosphate buffered at pH 7.3), without being allowed to come in contact with an air-water interface.

Protein was extracted in the medium used by Gregg and Ballentine (1946) (0.65% NaCl in 0.01 *M* phosphate buffer at pH 7.3), 65 ml. of solution being used for the 3410 eggs. The suspension was homogenized by hand for five minutes at 3–4° C. in a Ten Broeck glass homogenizer. A drop of the homogenate was examined under the microscope and no intact cells were noted.

The homogenate was kept at 4° C. for sixteen hours, with frequent shakings, then centrifuged at 600 G and the cloudy supernatant collected. Nutrient and blood agar plates streaked with the extract showed slight bacterial contamination. Therefore, 0.02 mg. Streptomycin ($CaCl_2$ complex) and 0.02 mg. Penicillin G potassium were added to each 50 ml. supernate to prevent bacterial growth and the extract was frozen in liquid air and stored at − 20° C. until use. The nitrogen

content of the extract, determined by micro-Kjeldahl, was 1.4 mg. N/ml. extract.

Twenty-four of 25 control eggs from the same clutch, and with jelly and vitelline membrane removed, developed normally to stage 22.

Preparation of antiserum. One male New Zealand Giant rabbit received eight subcutaneous injections, 0.5 ml. extract per injection, every fourth day (Cooper, 1948). A second uninjected male served as control. Seven days after the last injection, the animals were bled by cardiac puncture and the sera recovered. Both sera were heated to 56° C. for 0.5 hours to inactivate complement. Serum obtained from the animal injected with gastrula extract will henceforth be called anti-gastrula serum.

Precipitation tests were set up by layering 0.1 ml. of undiluted extract and of 11 two-fold dilutions (1:2 to 1:2048 in saline) over 0.1 ml. of antiserum, or over saline or normal serum as controls, in 10 × 75 mm. serological tubes. All tubes were incubated at 37° C. for one hour and then examined for presence of a precipitate ring at the interface. The antiserum produced rings of antigen-antibody complex at all antigen dilutions down through 1:1024. Controls were negative.

Tests of antiserum effects on reaggregation. For experiments dealing with ectoderm and endoderm, the membranes were removed with fine forceps from stage 10 or 11 *Rana pipiens* embryos, or the dorsal lip stage of *Triton alpestris*, and the desired tissues were isolated with glass needles in Holtfreter's solution. If mesoderm was to be used in addition to ectoderm and endoderm, the tissues were isolated either from stage 12 *Rana pipiens* embryos or from the dorsal lip stage of *Triton alpestris*.

The isolates were transferred by pipette to a syracuse dish containing 10 ml. sterile Holtfreter's solution, with 2% agar as substratum, and 1% KOH was added dropwise until disaggregation was complete. Occasionally, a clear viscous, somewhat elastic material or slime could be seen in the disaggregate; such preparations were rejected with one exception. The cells were washed 4–6 times with sterile Holtfreter's solution. Finally 10 ml. of normal serum or anti-gastrula serum, diluted with distilled water to give a final salt concentration of 0.38%, were added. By use of glass needles the cells were pushed into a heap with ectoderm, mesoderm, and endoderm cells randomly distributed.

Results

Twenty different combinations of tissues, isolated cells, antiserum, normal serum, and Holtfreter's solution were examined.

Effect of Rana pipiens anti-gastrula serum on disaggregated whole Rana pipiens embryos. Stage 9, 10 and 12 embryos were disaggregated and placed in anti-gastrula serum. After 1.5 hours the reaggregation, if any, was slight. At 7–14 hours the cells had adhered and sorted out to some degree, but remained spherical with conspicuous intercellular spaces. Endoderm cells in particular were loosely packed. Some slime was noted between the cells. In the stage 12 disaggregate, it appeared as though ectoderm cells would adhere to ectoderm, but not to endoderm, when pushed into contact. Endoderm cells, on the other hand, would adhere to endoderm, but not to ectoderm.

In contrast, the cells of disaggregates of stage 9 and 12 embryos, in *normal* serum, adhered to each other at the end of four hours, and the aggregate began

to round up. At eight hours, the aggregates were well rounded with interspersed patches of ectoderm and endoderm cells forming a smooth surface. At 15 hours the coat was being reconstituted. No masses of slime were noted.

Effect of Rana pipiens anti-gastrula serum on disaggregated ectoderm, endoderm, and mesoderm cells of Rana pipiens. After one hour in anti-gastrula serum, about 100 disaggregated mixed ectoderm, mesoderm, and endoderm cells, from a stage 12 embryo, had partially adhered. The ectoderm and endoderm cells appeared to have sorted out, but were loosely packed. No change was noted after this period.

Effect of antiserum on disaggregated ectoderm and endoderm cells of Rana pipiens. After 3.5 hours in antiserum, approximately 100 disaggregated ectoderm and endoderm cells, from a stage 11 embryo, adhered to each other, but the aggregate failed to round up. At four hours, the cells remained spherical and the aggregate surface was not smooth. Ectoderm and endoderm cells appeared randomly dispersed. No slime was noted. At 65 hours the ectoderm and endoderm cells still had not sorted out, and the surface was unchanged.

Ectoderm and endoderm cells of stage 11, after one hour in *normal* serum, had adhered and the surface was becoming smooth. By four hours, the aggregate had rounded up and ectoderm and endoderm cells had sorted out. The cells at the surface were flattened and a smooth exterior was noted at 17 hours. By 65 hours, the ectoderm and endoderm formed two separate aggregates connected by a short stalk of cells.

Effect of Rana pipiens anti-gastrula serum on disaggregated endoderm and ectoderm cells of Triton alpestris. Approximately 130 ectoderm and endoderm cells of the dorsal lip stage of *Triton alpestris* were disaggregated and placed in *Rana pipiens* anti-gastrula serum. After one hour, the cells had adhered to each other, with surface cells flattened and in intimate contact. At 17.5 hours the aggregate had become spherical with smooth surface. No slime was noted. At six

TABLE 1

Summary of experiments showing effects of frog anti-gastrula serum on reaggregation of dissociated frog and salamander cells

	Mixtures of disaggregated ectoderm and endoderm cells				Mixtures of disaggregated ectoderm, mesoderm, and endoderm cells			
	Normal serum		Antiserum		Normal serum		Antiserum	
Condition	2R*	1T**	1R	1T	3R	1T	5R	1T
Time (hours)	17	17	28	18	8	16	14	16
Aggregates rounded:	+	+	−	+	+	+	−	+
Exposed cell surfaces are flattened:	+	+	−	+	+	+	−	+
Random intermixture of ectoderm and endoderm cells:	−		+		−		±	
Time (hours)	65	144	65	144	36	144	36	144
Ectoderm and endoderm in separate aggregates:	+	+	−	+	−	−	−	−

* Number of experiments with cells from *Rana pipiens* embryos (R).

** Number of experiments with cells from *Triton alpestris* embryos (T).

days the ectoderm and endoderm cells had sorted out to form two completely separate aggregates.

Ectoderm and endoderm cells in *normal* serum sorted out after six days to form two aggregates connected by a short stalk.

Effect of Rana pipiens anti-gastrula serum on disaggregated mesoderm, ectoderm, and endoderm cells of Triton alpestris. About 130 ectoderm, mesoderm, and endoderm cells of *Triton alpestris*, dorsal lip stage, in *Rana pipiens* anti-gastrula serum formed smooth surfaced aggregates after one hour and the aggregate became spherical by 16 hours. No slime was noted. Cells in *normal* serum behaved in identical fashion.

The results, summarized in Table I, indicate strongly that, in embryonic amphibian cells as in adult sponge cells, specific surface antigens are an essential part of the mechanism by which adjacent cell surfaces are bound together during reaggregation.

Discussion

The results with embryonic amphibian cells are difficult to explain except in terms of the Tyler-Weiss hypothesis that contiguous cell surfaces are normally held together by forces similar to those between antigens and homologous antibodies (Tyler, 1940, 1942, 1946, 1947; Weiss, 1941, 1947, 1950).

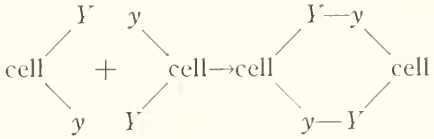
As in the case of cell adhesion in sponges described by Spiegel (1954) the results show that reaggregation of embryonic cells is inhibited, in contrast to the usual phenomenon of agglutination, by the presence of homologous antibodies. Spiegel (1954) has presented three conditions under which such inhibition may occur: (1) extreme antibody excess; (2) univalent antibodies; (3) structure of cell surface. Only the third condition will be discussed here (see Spiegel, 1954, for a discussion of conditions 1 and 2).

It has been suggested by Professor Albert Tyler that perhaps the cell surface of embryonic amphibian cells (and also of sponge cells) is a flexible, folded structure with the specific surface antigens so situated that the two or more valence groups of a *single* antibody molecule would tend to react with antigen on the *same* cell. Only a few, if any, valence groups would be free to react with receptor sites of other cells and thus would account for the absence of agglutination. It is, of course, also assumed that the reaction between specific surface antigens either blocks the adhesion sites or in some manner (*i.e.*, steric interference) interferes with them. The interpretation of the failure of the antibodies to agglutinate the sponge cells or the embryonic amphibian cells is a modification of the hypothesis presented by Coombs *et al.* (1951) to account for failure of homologous multivalent antibodies to agglutinate the red cells of most oxen. In their view a deep location of the antigens is assumed so that a multivalent antibody molecule after combining with one cell cannot reach receptors of a second cell.

That these surface antigens are specific has been demonstrated by the ability of cells of *Triton alpestris* embryos to reaggregate normally in *Rana pipiens* anti-gastrula serum.

The Tyler-Weiss hypothesis, as interpreted by Spiegel (1954), implies that adhesion depends on the presence, in the cell membranes, of two intra-molecular configurations with the reciprocal structural relationship of antigen and antibody. These exist either within a single molecular species or in distinct substances. If

the two reciprocal configurations are designated by Y and y, then the *unit* reaction in cell adhesion would be



As a logical consequence of this hypothesis, the segregation of ectoderm and endoderm cells from each other, in Holtfreter's solution or normal serum, into two separate aggregates, would indicate that different surface antigens are present on cells of each of the two tissues. Recent evidence obtained by Clayton (1953) with *Triton alpestris* embryos, seems to indicate that ectoderm and mesoderm, at least, contain different antigens. However, endoderm antigens were not investigated and the location of these antigens in the cell was not determined.

The fact that mixtures of ectoderm, endoderm, *and* mesoderm cells, in Holtfreter's solution or normal serum, round up to form a single aggregate consisting of three concentric layers of cells, with the mesoderm layer situated between ectoderm and endoderm (as in normal development), suggests an interesting possibility with regards to the surface antigens of mesoderm. Perhaps mesoderm, in addition to possessing specific surface antigens which are important for the adhesion of mesoderm cells to each other, also possesses sites which are complementary to ectoderm surface antigens *and* to endoderm surface antigens. Thus, in effect, mesoderm would act as a heterovalent antibody (a multivalent antibody molecule, with *different reactive sites,* which are capable of combining with at least two different antigenic groups) to ectoderm cells and to endoderm cells, and would be capable of binding the two cell types together. This would be analogous to the hypothesis proposed by Spiegel (1954) for the failure of segregation of mixtures of the cells of two species of sponge in antiserum produced in rabbits by simultaneous injection of cells of *both* species. Here it was proposed that *the rabbit* formed heterovalent antibody to the surface antigens of cells of both species and thus such antibody molecules were able to agglutinate both cell-species.

Each of the amphibian results is what would be expected from the sponge experiments performed by Spiegel (1954). Amphibian and sponge experiments, together, can be explained on the basis that the ability of cells to recognize each other depends upon the presence, in their surfaces, of specific antigens which also bring about the normal adhesion of cells, by reciprocal reactions similar to those between antigens and their homologous antibodies.

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Summary

An investigation of cell adhesion in embryonic amphibian cells is reported here. Antiserum was made in rabbits vs. a cell extract of stage 12 *Rana pipiens* embryos. The reaggregation of dissociated cells of Rana ectoderm and endoderm was in-

hibited by the antiserum. The antiserum had no effect on the reaggregation and segregation of dissociated cells of *Triton alpestris* embryos. The role of antigen-antibody-like reactions in cell adhesion is discussed.

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